Novel peptides derived from α_{s1}-casein with opioid activity and mucin stimulatory effect on HT29-MTX cells

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ABSTRACT

This study investigates the opioid effect of α_{s1}-casein fragments related to ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149}, which had previously shown mucin-stimulatory activity in human goblet cells. Peptides ^{144}YFYPEL^{149} and ^{144}YFYPE^{148} showed opioid agonistic activity in guinea pig ileum, and in mouse vas deferens but to a lower extent. Peptides were partly hydrolysed during the assay and several of the resulting fragments lost the N-terminal Tyr residue. Docking of peptides ^{144}YFYPEL^{149} (active) and ^{144}YFYP^{147} (inactive) into the active site of the opioid receptor model showed remarkable differences regarding the flexibility at the third intracellular loop of the receptor and the interaction with Pro at the peptide C-terminus that forced residues Arg^{148} and Glu^{166} from the receptor to move towards the interior of the binding pocket. The study on human cells HT29-MTX has shown that α_{s1}-casein ^{144}YFYPEL^{149} is the minimum fragment able to stimulate MUC5AC expression.

KEYWORDS: Opioid peptide; mu-opioid receptor; molecular dynamics; mucin; goblet cells

HIGHLIGHTS:

^{144}YFYPEL^{149} and ^{144}YFYPE^{148} demonstrated opioid activity on guinea pig ileum

^{144}YFYPEL^{149} and ^{144}YFYP^{147} presented different behaviour against opioid receptor

^{144}YFYPEL^{149} was the minimum fragment with stimulatory effect on MUC5AC expression

1. INTRODUCTION

During food digestion, proteins render a large variety of different peptides. Some of these sequences are structurally similar to endogenous physiologically active peptides and therefore, these food-derived sequences can interact with the same receptors in the organism, exerting an agonist or antagonist activity. One of the best examples where this applies, are food-derived opioid peptides, i.e., exogenous opioid receptor ligands with agonistic activity. From milk, peptides derived from β-casein, referred as β-casomorphins, were the first food protein-derived opioid receptor ligands whose sequences were identified (Brantl, Teschemacher, Henschen, & Lottspeich, 1979). They have been found in *in vivo* digestion products from humans and minipigs (Barbé et al., 2014; Boutrou et al., 2013). The common structural characteristics of both, exogenous and endogenous opioid peptides, are the presence of a tyrosine residue at the N-terminus and the presence of other aromatic residue, phenylalanine or tyrosine, in the third or fourth position (Meisel, 1997). In case of the α_{s1}-casein derived peptides or α-casein exorphins, the active sequence can contain an additional arginine residue at the N-terminus. Although caseins are source of many peptides showing agonist or antagonist action on different opioid receptors, opioid peptides from other protein sources have been also described, such as whey proteins (Antila et al., 1991) and hemoglobin (Zhao, Garreau, Sannier, & Piot, 1997) from animal sources and gluten, rice, or soy from plant proteins (Yoshikawa, 2015).

Biological activities observed for these food-derived opioid sequences include analgesia and modulation of social behaviour, after parenteral or intracerebral administration to animals. Orally administered food-derived opioid peptides have demonstrated to influence postprandial metabolism by stimulating secretion of insulin and somatostatin, prolongation of gastrointestinal transit time, stimulation of food intake, and effects on the immune system, among others (for reviews regarding biological activity, see Meisel & FitzGerald, 2000; Rutherfurd-Markwick, 2012; Teschemacher, 2003; Teschemacher, Koch & Brantl, 1997).

Moreover, it was found that the opioid peptide β-casomorphin-7 (^{60}YPFPGPI^{66}) induced intestinal mucin release through a nervous pathway and opioid receptor activation in *ex-vivo* preparations of rat jejunum (Claustre et al., 2002; Trompette et al., 2003). In human (HT29-MTX) and rat (DHE) intestinal mucin-producing cells, this peptide increased secretion and expression of mucin, and these effects were prevented with the μ-opioid antagonist cyprodime (Zoghbi et al., 2006). Similar effects were reported for the μ-opioid ligands, α-lactorphin (Martínez-Maqueda et al., 2012) and β-lactorphin (Martínez-Maqueda, Miralles, Ramos, & Recio, 2013b). However, other β-casein-derived peptides whose structures do not fulfil the requirements of opioid ligands have also demonstrated regulation of mucin production in HT29-MTX cells and in animals, such as the peptide β-CN f(94-123) found in yogurts and the derived fragments (94-108) and (117-123) (Plaisancié et al., 2013; Plaisancié et al., 2015).

Our group had shown the mucin secretory activity of various bovine α_{s1}-casein-derived peptides with favourable structures to bind opioid receptors due to the presence of Tyr at the N-terminus or in the second position and other Tyr in the third or fourth position. From these peptides, fragments ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149} and a casein hydrolysate containing both sequences produced an increased mucin secretion and MUC5AC gene expression in HT29-MTX cells (Martínez-Maqueda, Miralles, Cruz-Huerta, Recio, 2013a). Interestingly, these two peptides had been identified in *in vivo* gastric and duodenal human digests after milk ingestion (Chabance et al., 1998). However, despite the favourable structure of these sequences and their potential to interact with opioid receptors located at the intestinal tract, the opioid activity of these peptides has not been previously demonstrated. The objective of this work was to investigate the opioid effect of the bovine α_{s1}-casein fragment ^{143}AYFYPEL^{149}, and four derived peptides comprising the core structure for opioid activity YFY in guinea pig ileum and mouse vas deferens preparations. A molecular docking of two peptides into the active site of the μ-opioid receptor was carried out to identify the key residues responsible for the affinity to

the receptor. In addition, a preliminary study of these sequences on MUC5AC gene overexpression in HT29-MTX cells is shown.

2. MATERIALS AND METHODS

2.1. Peptides

α_{s1}-casein fragments ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149}, ^{144}YFYPE^{148}, ^{144}YFYP^{147} and ^{144}YFY^{146}, and the β-casein fragment ^{60}YPFPGPI^{66} were synthesized in house using conventional solid-phase FMOC synthesis with a 433A peptide synthesizer (Applied Biosystems, Warrington, UK). In the peptide purification protocol, the treatment with acetic acid was included to replace trifluoroacetic acid which affects the pH of the peptide solutions in the bioassays. Their purity (>90%) was verified in our laboratory by reverse phase high performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS).

2.2. Isolated preparations from guinea pig ileum and mouse vas deferens

Female guinea-pigs weighing 300-450 g, and male CD-1 mice weighing 25-60 g were used. Myenteric plexus-longitudinal muscle strips (MP-LM) from guinea pig ileum, and the mouse vas deferens were isolated as described by Ambache (1954) and Hughes et al. (1975), respectively. Tissues were suspended in a 10 ml organ bath containing 5 ml of Krebs solution (NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19; $MgSO_4$ 1.2; $NaHCO_3$ 25; glucose 11mM). This solution was continuously gassed with 95% O_2 and 5% CO_2. Tissues were kept under 1 g (guinea pig ileum) or 0.5 g (mouse vas deferens) of resting tension, at 37 ^{o}C and were electrically stimulated through two platinum ring electrodes. MP-LM strips were stimulated with rectangular pulses of 70 V, 0.1 ms duration and 0.3 Hz frequency, and mouse vas deferens with trains of 15 rectangular pulses of 70 V, 15 Hz and 2 ms duration each min. The isometric force was monitored by computer using a MacLab data recording and analysis system. In both

assays the interval between applications of increasing concentrations was optimized to obtain a stable signal, and it was 9 min for α_{s1}-casein peptides, and 3 min when control opioid agonists were tested. To evaluate the opioid-agonistic activity of peptides in the guinea pig ileum, cumulative concentration-response curves with five doses in the range 6.1×10^{-8} – 1.0×10^{-5} M were constructed in a step by step manner as follows: after 15 min-stabilisation of MP-LM strips in organ bath, electrical stimulation was applied, and peptide´s effect on the electrically induced contractions was evaluated once the response reached a plateau. Morphine was used as μ-opioid agonist positive control. To corroborate that the inhibitory effect of the peptides was mediated through interaction with opioid receptors, one dose of naloxone (1.0×10^{-6} M, Sigma), an non-selective opioid antagonist, was added to the organ bath at the end of each experiment. In mouse vas deferens preparations, non-cumulative concentration-response curves with four doses in the range 5.5×10^{-7} – 1.0×10^{-5} M were tested. Once tissue stabilisation, the first peptide dose was added and evaluated. The tissue was then washed, and subsequent doses were applied. In this preparation, [D-Pen(2),D-Pen(5)]-enkephalin (DPDPE) was used as δ-opioid agonist, and one dose of naltrindole (1.0×10^{-9} - 1.0×10^{-7} M, Sigma), a δ-selective opioid antagonist, was added after the experiments to evaluate selective interaction with δ-opioid receptors. Results were expressed as % of inhibition, taking the mean amplitude of the last five contractions before the addition of the peptides as 100%. In guinea pig ileum, when the effect of peptidase inhibitors (captopril, amastatin and phosphoramidon 1.0×10^{-6} M, Sigma) was evaluated, they were added 5 min before the beginning of the peptide´s concentration-response curve (Akahori et al., 2008). Each tissue was employed only once, with at least six biological replicates per condition. Experimental protocols used in this investigation were approved by the Ethical Committee of Rey Juan Carlos University. Data were analyzed using GraphPad Prism 5.03 software by a one-way ANOVA, followed by the Bonferroni Multiple Comparison test. Differences between each dose with a *P* value < 0.05 were considered significant.

2.3. Analysis by HPLC-MS/MS

In order to evaluate the stability of the peptides during the activity assay on MP-LM preparations, they were added at 5.0×10^{-6} M to the organ bath solution, and aliquots were withdrawn at time 0, 4, and 9 min, frozen immediately and kept at − 20 ^{o}C until analysis. Analysis of the peptides was performed using an Acquity liquid chromatography system (Waters) connected to microToF II Quadrupole-Time-of-Flight mass spectrometer (Bruker). A Waters Acquity UPLC BEH C_{18} column 100 mm length, 2.1 mm internal diameter, 1.7 µm packing was used. A binary elution gradient, based on water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid (v/v), and operated at a flow rate of 0.2 mL/min, was adopted. A gradient elution program based on solvent B increase from 0% to 70% in 30 min was used. Column reconditioning at the initial mobile phase composition was accomplished in 15 min. ESI-MS acquisitions were carried out in positive ion mode. The LC-MS system was controlled by the HyStar 3.2 software (Bruker). The main parameters of the ESI interface and of the quadrupole were: Dry temperature 180 ^{o}C; Dry gas flow 4.0 L/min; Nebulizer gas pressure 0.4 bar; Capillary voltage 4.5 kV; Quadrupole Ion Energy 5.0 eV. The ToF analyzer was calibrated on a daily basis in MS mode, using the *m/z* ratios of adduct ions arising from sodium formate as reference.

2.4. Molecular modelling receptor-peptide

Homology model of the µ-opioid receptor (UniProtKB - P35372 (OPRM_HUMAN)) was built by standard homology modeling techniques using MODELER version 9.7. (Eswar et al., 2006), as described in the Supplementary Data. The structure of the human δ- and µ-opioid receptors (Protein Data Bank entries 4RWA and 5C1M) (Fenalti et al., 2015; Huang et al., 2015) were used as template. The structure of the bifunctional δ-opioid antagonist and µ-opioid agonist tetrapeptide Dmt-Tic-Phe-Phe-NH_2 (Protein Data Bank entry 4RWA) was used as

template for docking the peptides ^{144}YFYPEL^{149} and ^{144}YFYP^{147} into the active site of the opioid receptor model.

2.5. Cell culture

HT29-MTX, a human colon adenocarcinoma-derived mucin-secreting goblet cell line was provided by Dr. Thécla Lesuffleur (Lesuffleur et al., 1993). The cell line was grown in plastic 75-cm^2 culture flasks in Dulbecco´s Modified Eagle´s medium supplemented with 10% fetal bovine serum and 10 mL/L penicillin-streptomycin solution (all from Gibco, Paisley, UK) at 37 ^{o}C in a 5% CO_2 atmosphere in a humidified incubator. Cells were passaged weekly using trypsin/EDTA 0.05% (Gibco). The culture medium was changed every two days. To study the effect of peptides, cells were seeded at a density of 5×10^5 cells per well in 12-well culture plates (Nunc, Roskilde, Denmark). The cell line was used between passages 12 and 19. Experiments were performed 21 days after confluence. The culture medium was replaced, 24 h before the studies, by serum- and antibiotic-free medium to starve the cells and to eliminate any interference from extraneous proteins or hormones. After serum-free medium removal, the monolayer was washed twice with PBS. Serum-free medium with or without peptide (5.0×10^{-5}, 1.0×10^{-4}, and 5.0×10^{-4} M) was added to the cells and incubated at 37 ^{o}C for 2 to 24 h in a 5% CO_2 humidified atmosphere. The supernatants were collected, frozen and stored at -80 ^{o}C. The total RNA was isolated with Nucleospin® RNA II (Macherey-Nagel, Düren, Germany) according to manufacturer´s instruction.

2.6. Quantitative RT-PCR assays (qRT-PCR)

qRT-PCR amplification was carried out using a Lightcycler 480 (Roche, Mannheim, Germany) in 384 wells microplates (Roche). RNA (375 ng) was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer´s instruction. For MUC5AC (accession no. AJ001402), target gene, primers 2870-2889/3109-3091 were used. For reference genes human cyclophilin (accession no. Y00052) and human β-actin

(accession no. NM_001101) primers 280-304/445-421 and 879-896/1076-1053, respectively, were used (Tai et al., 2008; Zoghbi et al., 2006). The SYBR Green method was used and each assay was performed with cDNA samples in triplicate. Each reaction tube contained 5 µL 2x SYBR Green real-time PCR Master Mix (Roche), 0.25 µL of a 10 µM of gene-specific forward and reverse primers, 0.27 µL of cDNA (5.06 ng), and 4.23 µL of water. Amplification was initiated at 95 ^{o}C for 5 min, followed by 45 cycles of 95 ^{o}C for 10 s, 60 ^{o}C for 10 s and 72 ^{o}C for 10 s. Control PCRs were included to confirm the absence of primer dimer formation (no-template control), and to verify that there was no DNA contamination (without RT enzyme). All real-time PCR assays amplified a single product as determined by melting curve analysis. Amplification efficiencies of the target and reference genes were performed, and the relative expression levels of the target gene were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Human cyclophilin and human β-actin were tested as reference gene. Human cyclophilin gene was then chosen to calculate the threshold cycles because it had previously been shown to be constant under all conditions used. All experiments were performed at least three times in triplicate. Data were analyzed using GraphPad Prism 5.03 software by a two-way ANOVA. For a better comparison of the concentration versus control data for each time, data were analyzed by one-way ANOVA, followed by the Newman-Keuls test. Differences between means and controls with a *P* value < 0.05 (*), or *P* value < 0.01 (**) were considered significant.

3. RESULTS

3.1. Activity of α_{s1}-casein fragment ^{143}AYFYPEL^{149} and derived peptides on guinea pig ileum and mouse vas deferens preparations

As illustrated in Figure 1, the inhibition of the electrically induced contractions in MP-LM preparations in cumulative curves of five doses showed a significative increase upon

addition of the α_{s1}-casein peptides ^{144}YFYPEL^{149}, and ^{144}YFYPE^{148} between the first and the third dose but the curve did not show any further increase. The other peptides, ^{143}AYFYPEL^{149}, ^{144}YFYP^{147} and ^{144}YFY^{146}, did not show inhibitory effect on this preparation. The maximum inhibition responses for ^{144}YFYPEL^{149} (35.1 ± 5.6%), and ^{144}YFYPE^{148} (31.8 ± 2.5%), were reached at a dose 5.5×10^{-7} M and it was followed by a smooth decrease. As positive controls, β-casomorphin-7 (YPFPGPI) and morphine were used. β-casomorphin-7 showed an increasing trend up to 1.0×10^{-5} M with a maximum response of 23.3 ± 5.1%, while the morphine inhibition curve increased up to 1.6×10^{-6} M reaching a 60.6 ± 5.8% of inhibition on the electrically stimulated contractions of the MP-LM preparations. In this latter case, the plateau observed for the highest concentration (5.0×10^{-6} M) could be due to receptor saturation. However, the decreasing trend of the curves obtained for the α_{s1}-casein-derived peptides from the third dose led us to consider the possibility of peptide degradation in the organ bath. In order to check peptide stability during the experiments, aliquots of the organ bath were withdrawn at time 0, 4 and 9 min and analysed by HPLC-MS/MS. The chromatographic areas of the extracted molecular ion corresponding to the assayed peptides showed a decrease with the time (Supplementary Figure 1). The observed decrease in the area of intact peptide was accompanied with the increase of derived peptide fragments, which could be also identified by their fragmentation pattern. In this way, from peptide ^{143}AYFYPEL^{149} increasing concentrations of fragments ^{144}YFYPEL^{149}, ^{145}FYPEL^{149}, and ^{146}YPEL^{149} were detected over time; from peptide ^{144}YFYPEL^{149}, fragments ^{145}FYPEL^{149}, ^{146}YPEL^{149}, and ^{144}YFYP^{147} were found; and from ^{144}YFYPE^{148}, the formation of ^{145}FYPE^{148} could be followed. No peptide fragments were detected for ^{144}YFYP^{147} or ^{144}YFY^{146}, although a concentration decrease ca. 34% and 29% was found from 0 to 9 min, respectively. For these two short peptides, it is possible that although peptide degradation could occur, the derived peptide fragments could not be detected due the short size of the fragments. It has to be noted that some of the new peptide fragments generated by the action of tissue peptidases preserved the cluster ^{144}YFY^{146}, but in other cases the N-

terminal Tyr residue was lost, and therefore, presumably their potential opioid effect. Since the lost in opioid effect along the accumulative dose-response curve could be attributed to the peptide degradation over time, the assays were repeated in the presence of three peptidase inhibitors.

Figure 2 shows the inhibition curves for peptides ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149}, ^{144}YFYPE^{148}, ^{144}YFYP^{147} and ^{144}YFY^{146} when peptidase inhibitors captopril, amastatin and phosphoramidon were used. The inhibition curves for all peptides, except ^{144}YFYP^{147}, showed an increasing trend with the peptide concentration and a significant dose-effect was found for various peptides, such as, ^{144}YFYPEL^{149} and ^{144}YFY^{146}. Peptide ^{144}YFYPE^{148} reached the highest mean inhibitory value, 31.6 ± 4.6%, although the inhibition percentages determined for ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149}, and ^{144}YFY^{146} were not significantly different to this, being 22.2 ± 4.1, 26.2 ± 4.9 and 25.5 ± 5.3%, respectively. The suppressive effect of these peptides on the electrically stimulated contractions in MP-LM preparations was reverted by the use of naloxone at 1.0×10^{-6} M, once the maximum concentration of the peptide was tested. In addition, the stability of the peptides over the time in the presence of peptidase inhibitors was also evaluated, as previously. As expected, derived peptide fragments were not detected or their intensities were considerably lower (data not shown).

In the mouse vas deferens preparation, α_{s1}-casein-derived peptides showed lower activity. Peptides ^{143}AYFYPEL^{149}, ^{144}YFYPEL^{149} and ^{144}YFYPE^{148} induced a maximum inhibitory effect of the contractions of 8.8 ± 3.4, 6.9 ± 1.6 and 13.4 ± 2.8%, respectively, at the highest dose assayed (1.0×10^{-5} M), with a dose-effect trend only found for ^{144}YFYPE^{148}. The positive control, DPDPE, also showed a dose-dependent activity, with the maximum inhibitory effect found at 6×10^{-8} M (55.3 ± 10.2 %). By the addition of naltrindole (1×10^{-9} M), a selective δ-opioid receptor antagonist, DPDPE effect was partly reversed (36.0 ± 7.9 %), while the weak activity found for the peptides was not affected.

3.2. Molecular modelling of the μ-opioid receptor bound to α_{s1}-casein peptides

To better understand the opioid activity of these α_{s1}-casein peptides and aiming to elucidate a structure-activity relationship, molecular dynamics simulations of peptides binding at μ-opioid receptor were performed. As representative examples, peptides ^{144}YFYPEL^{149} and ^{144}YFYP^{147} were chosen for these experiments to explain the lack of activity of the shorter form, according to results on guinea-pig ileum preparations.

The structural stability of the complex receptor-peptide was evaluated by monitoring the Root-Mean-Square Deviation (RMSD) between different structures sampled along the simulation. The fluctuations of the individual residues from the receptor and the peptides were studied. In both cases, intra and extra loops of the receptor exhibited the greatest variability, most noteworthy loops between residues 145-150, 195-200, 240-250 and the C-terminal domain of the receptor (Figure 3A). However, when peptide ^{144}YFYP^{147} was assayed the loop between residues 195-200 started the simulation with a retracted loop but it became stretched maintaining this position for the rest of the simulation (Figure 3B). While peptide ^{144}YFYPEL^{149} stays stable at the active site at its first three residues and fluctuates due to the repositioning of its last two residues, movements of peptide ^{144}YFYP^{147} are mainly due to residues Tyr at the third position and Pro at the C-terminus, as consequence of the new C-terminal group.

As shown in Table 1 and supplementary Figure 2 the interaction energy of the different amino acid residues of the peptides at the active site of μ-receptor was calculated. The position of peptide ^{144}YFYPEL^{149} at the base of active site is stabilized by strong van der Waals, hydrogen bond, and charge-charge interactions. This is clearly seen at the final model where the interactions of the residues of the receptor Asp^{84}, Tyr^{263}, His^{234}, and Trp^{230} interact with Tyr at the peptide N-terminus stabilizing this position (Figure 4A). Peptide Phe and Tyr residues, at the second and third position, respectively, showed strong hydrogen bonds and van der Waals

interactions between their aromatic rings and compatible μ-receptor residues that also help N-terminal Tyr to maintain its right configuration (Figure 4B and 4C). At the top of the cavity, peptide Glu and Leu establish charge-charge and hydrogen bonds interactions, building a network of electrostatic interactions between the peptide, some water molecules, and the last residues of the cavity (Figure 4D). In the case of peptide ^{144}YFYP^{147}, the behaviour of the N-terminal Tyr at the right side of the binding pocket resembles that described above for ^{144}YFYPEL^{149} (Figure 4E). However the interaction between the Tyr at the third position and the Gln^{61} residue from the receptor changes from van der Waals-type to hydrogen bond-type (Table 1). Moreover, an additional hydrogen bond is established with Tyr^{85}, instead of Tyr^{12} as before. Consequently, as shown in Figure 4G, the orientation of the second Tyr residue from ^{144}YFYP^{147} differs from that of peptide ^{144}YFYPEL^{149}. In addition, the C-terminal Pro residue from ^{144}YFYP^{147} played a remarkable role in receptor-peptide relation, not described in the case of ^{144}YFYPEL^{149}, by establishing a strong charge-charge-type interaction with Arg^{148}, which modifies the configuration of this location (Table 1 and Figure 4H).

The interactions of peptide-receptor residues were monitored during the simulation time (45 ns). In case of peptide ^{144}YFYPEL^{149}, interactions with N-terminal Tyr were stable during the simulation, whereas for the rest of residues interactions became stronger as the simulation progressed (Supplementary Figure 3). However, main interactions of peptide ^{144}YFYP^{147} seem to follow a different behaviour. Whereas Tyr at the peptide N-terminus and Asp^{84} maintained a quite stable interaction, this peptide residue lost interaction strength with Tyr^{85} after 10 ns, and with Tyr^{263} and His^{234} at the last steps of the simulation. It is remarkable that the second peptide Tyr residue began to show a tough interaction with Tyr^{85}, Asp^{84}, and Ile^{81} at the opioid receptor after 20 ns. Similarly, C-terminal Pro increased interaction with Arg^{148} and Thr^{155} at the end of the simulation. All these modifications might be most likely due to a change in the peptide-receptor orientation.

3.3. Activity of bovine α_{s1}-casein-derived peptides ^{144}YFYPE^{148}, ^{144}YFYP^{147} and ^{144}YFY^{146} on MUC5AC expression

Because peptides ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149} had previously demonstrated stimulatory effect on mucin secretion and MUC5AC gene expression (Martínez-Maqueda et al., 2013a), the effect of the shorter derived peptides on HT29-MTX cells was also evaluated. The relative expression of MUC5AC was determined by qRT-PCR after exposure of HT29-MTX cells to the peptides. Table 2 shows the maximum relative MUC5AC expression level along 24 h of treatment with ^{144}YFYPE^{148}, ^{144}YFYP^{147}, and ^{144}YFY^{146} at three concentrations (5.0×10^{-5}, 1.0×10^{-4}, and 5.0×10^{-4} M). Although treatment with ^{144}YFY^{146} showed increased levels of MUC5AC after 24h at the three concentrations, these values did not reach significance due to the high variability between replicates. For the other two peptides ^{144}YFYPE^{148}, ^{144}YFYP^{147}, no significant MUC5AC over-expression was observed compared with controls (untreated cells). From these two latter peptides, only ^{144}YFYPE^{148}, but not ^{144}YFYP^{147}, had demonstrated the ability to inhibit electrically stimulated contractions in MP-LM. According to these results, the assayed shorter forms exhibited different behaviour on the mucin gene expression in HT29-MTX cells than the longer peptides ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149}.

4. DISCUSSION

β-casomorphins were the first food-derived opioid peptides and were found in a casein hydrolysate in 1979 (Brantl et al., 1979). Since then, different opioid peptides derived from milk proteins and other food proteins have been described. The present study was undertaken to examine if two α_{s1}-casein-derived sequences, ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149}, which share some structural characteristics with previously described opioid peptides, could also exert this effect. These two sequences, found in a peptic casein hydrolysate, had also demonstrated to stimulate mucin secretion and gene expression in HT29-MTX cells (Martínez-Maqueda et al.,

2013a), and it had been suggested that this effect could be mediated by interaction with μ-opioid receptors (Zoghbi et al., 2006). The results of this study showed, for the first time, opioid activity in the guinea pig MP-LM preparation for various related α_{s1}-casein sequences, especially ^{144}YFYPEL^{149} and ^{144}YFYPE^{148}. Other related fragments, ^{143}AYFYPEL^{149} and ^{144}YFY^{146} were able to exert this effect, but only when peptidase inhibitors were added to the preparation.

In view of their structures, opioid peptides have been divided into two groups designated as “typical” and “atypical” (Teschemacher et al., 1997). “Typical” opioid peptides exhibit the definite N-terminal sequence YGGF, characteristic of the endogenous opioid peptides: enkephalins, endorphins, and dynorphins. “Atypical” peptides are characterized by the presence of Tyr at the N-terminus which is a structural motif important in ligand-receptor binding, although this N-terminal residue is not present in nociception/orphanin FQ (Reinscheid et al., 1995). The α_{s1}-casein derived peptides studied in this work share some structural characteristics with previously described α_{s1}-casein exorphins. Loukas, Varoucha, Zioudrou, Streaty, and Klee (1983) identified various α_{s1}-casein opioid peptides, ^{90}RYLGYLE^{96}, ^{90}RYLGYL^{95} and ^{91}YLGYLE^{96} from a digest of casein with pepsin. Similarly to α_{s1}-casein exorphins, peptides in this work were identified in a peptic casein hydrolysate and also possess two Tyr residues, in our case separated by a Phe residue instead by the dipeptide LG, as occurs in the peptides previously described. In addition, some α_{s1}-casein exorphins are characterized by the presence of an additional Arg residue at the N-terminus. Our results show by comparison of the activity of ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149} that the presence of an additional Ala residue at the N-terminus diminishes the effect on the guinea pig ileum preparation, when no peptidases inhibitors were used. However, in presence of peptidases inhibitors ^{143}AYFYPEL^{149} exerted certain activity, and therefore, the lack of activity in absence of peptidase inhibitors might be explained by a rapid peptide degradation.

Peptides in this study, especially ^{144}YFYPEL^{149} and ^{144}YFYPE^{148}, showed naloxone-antagonizable inhibitory effect in the electrically stimulated guinea pig ileum preparation. This isolated tissue possesses both, μ- and κ-opioid receptors that regulate the contractile response when stimulated by inhibiting acetylcholine release during electrical stimulation of the myenteric plexus neurons. Although this preparation also contains cannabinoid, adenosine and adrenergic receptors (Taylor, 2011), the reversion of the effect by naloxone, a competitive antagonist at μ, δ, and κ-opioid receptors, proved the participation of these receptors on the effect of the assayed peptides. However, lower effects were found in the mouse vas deferens. This tissue can be used to study all three opioid receptor subtypes, but the expression of μ- and κ-opioid receptors is lower in this preparation than in guinea pig ileum (Taylor, 2011), being the δ-opioid the predominant receptor subtype (Lord, Waterfield, Hughes, & Kosterlitz, 1977). The activity of the assayed peptides in mouse vas deferens was not reverted by the use of naltrindole which suggests poor affinity for δ-opioid receptors. Likewise, bovine β-casomorphins have been also demonstrated to exert a higher opioid activity in the guinea pig ileum than in the mouse vas deferens assay (Brantl, Teschemacher, Bläsig, Henschen, & Lottspeich, 1981). On the contrary, the previously described α_{s1}-casein exorphins demonstrated a moderated effect on inhibition of adenyl cyclase in neuroblastoma × glioma hybrid cells, as well as, inhibition of contractions in mouse vas deferens, suggesting selectivity for δ-opioid receptors (Loukas et al., 1983). The assayed peptides show structural homologies with β-casomorphins, with an aromatic residue at the first and third position and a proline residue at the fourth position from the N-terminal. This is in agreement with the higher activity found in the guinea pig ileum preparation. Therefore, further studies with selective opioid antagonists such as β-funaltrexamine, naltrindole, and nor-binaltorphimine for μ-, δ-, and κ-opioid receptors, respectively, are required to evaluate the selectivity of the active peptides found in this study on different receptor subtypes.

Regarding activity, these peptides containing the tripeptide YFY were found to exert an agonist opioid effect on guinea pig ileum preparations, although moderate in strength. However, it has to be pointed that under our experimental conditions, i.e., application of increasing concentrations at 9 min intervals, peptide β-casomorphin-7, which was used as positive control, did not reach the activity values previously reported in guinea pig ileum preparations, with IC_{50} values between 3.2×10^{-6} and 6.9×10^{-6} M (Jinsmaa & Yoshikawa, 1999; Koch, Wiedemann, & Teschemacher, 1985; Yoshikawa, Fumito, Takashi, & Hideo, 1986). The time intervals used in this study were optimized to ensure signal stability. In addition, the stability of the assayed peptides in the organ bath was studied by HPLC-tandem mass spectrometry. It was demonstrated that these peptides were partly hydrolysed by the action of ileum peptidases within 9 min and some of the generated fragments lost the N-terminal Tyr residue characteristic of opioid peptides. In fact, it has been reported that endogenous opioid peptides, with homologous structure than the assayed peptides, are particularly sensitive to rapid hydrolysis by a number of peptidases that are present in the guinea pig ileum, as well as in other tissues (McKnight, Corbett, & Kosterlitz, 1983). From our results, it was also shown that the shape of the inhibition curve changed by the use of the three peptidase inhibitors but the maximum inhibitory activity did not significantly increase (Figure 1 vs. Figure 2). The change in the accumulative inhibition curves, compared to experiments conducted in absence of peptidase inhibitors confirmed that the decrease observed at higher doses in the absence of peptide inhibitors is caused by the peptide degradation during the assay. The combination of these peptidase inhibitors (captopril, amastatin and phosphoramidon 1.0×10^{-6} M) was not able to change the activity of morphine, DAMGO and metabolically stable synthetic opioid peptides in this preparation (Hiranuma et al., 1998). Aoki, Kajiwara, and Oka (1984) and McKnigh et al. (1983) demonstrated that the peptidase inhibitors mixtures captopril 1.0×10^{-6}, bestatin 10.0×10^{-6}, and thiorphan 1.0×10^{-6} M; and captopril 10.0×10^{-6}, bestatin 10.0×10^{-6},

thiorphan 0.3 × 10^{-6}, and L-Leu-L-Leu 2.0 × 10^{-3} M, respectively, did not affect, alone nor in combination, the contraction of the guinea pig MP-LM.

In order to explain the different opioid activity found for these structurally similar peptides, molecular dynamic simulations were performed with ^{144}YFYPEL^{149} which showed opioid activity in guinea pig ileum and ^{144}YFYP^{147} which was inactive. The homology model of μ-opioid receptor with the peptides docked at the active site was evaluated by the study of the energy stability and the identification of those responsible binding-residues at the cavity. The simulations with both peptides showed a notably energy variability in intra- and extra-cellular loops of the receptor at positions 145-150, 195-200, 240-250 and the C-terminal domain. This structural flexibility is in agreement with previous studies with the μ-opioid receptor (Serohijos et al., 2011). Similarly, it has been found that morphine leads to a greater flexibility of the third intra-cellular loop of the receptor, which is consistent with the critical role of this loop as the docking site of G-proteins binding (Waldhoer, Bartlett, & Whistler, 2004). Due to the flexibility observed in our simulation, it can be suggested that peptide ^{144}YFYPEL^{149} behaves as an opioid agonist. For peptide ^{144}YFYP^{147}, the flexible areas of the receptor were similar, but a remarkable difference was found at the movement of the loop 195-200, which is related to the activity of the receptor. In addition, while peptide ^{144}YFYPEL^{149} evoked a continuous change in this loop during the simulation, with peptide ^{144}YFYP^{147} it became fixed. Moreover, it has been found that both peptides establish two strong hydrogen bonds and a charge-charge interaction, in the same way as the morphinan agonist BU72 (Huang et al., 2015), between the N-terminal Tyr at the peptide and Tyr^{263}, His^{234}, and Asp^{84} residues. However, and in contrast with the ^{144}YFYPEL^{149} simulation, the second Tyr residue and Pro from ^{144}YFYP^{147} were found to create different interactions with the receptor, resulting in a clear change between the locations of both peptides at the active binding site. Besides, another fact that might contribute to the different behaviour is the orientation of Arg^{148} and Glu^{166} receptor residues. When peptide ^{144}YFYP^{147} was bound to the receptor, Arg^{148} and Glu^{166} residues had to move

towards the interior of the binding pocket to interact with C-terminal Pro of ^{144}YFYP^{147}. This process would require a large amount of energy due to their charged character, and when exposed to the solvent, would be highly solvated. The energy needed to remove this solvation shell might render peptide ^{144}YFYP^{147} inactive.

Previously, Zoghbi et al. (2006) had reported the activity of β-casomorphin-7 on mucin production in HT29-MTX cells via μ-opioid interactions. Given the effect observed in guinea pig ileum preparations, it was expected that in addition to ^{143}AYFYPEL^{149} and ^{144}YFYPEL^{149}, peptide ^{144}YFYPE^{148} could also induce MUC5AC gene expression. However, although this peptide at 1.0 × 10^{-4}, and 5.0 × 10^{-4} M showed increased expression levels over the control, it did not reach significance. There is sufficient literature that illustrates the lack of correlation between μ-opioid potency and effect on mucin secretion or gene overexpression. For instance, in a screening of several peptides with proved or probable μ- and δ-opioid activity, six out of eight peptides induced mucin secretion in HT29-MTX cells (Martínez-Maqueda et al., 2012). The evaluation of the effect on MUC5AC expression by α-lactorphin-amide and human β-casomorphin-5 revealed that only the first provoked a significant gene overexpression, despite both had behaved as mucin secretors (Martínez-Maqueda et al., 2012), being β-casomorphin-5 more potent than α-lactorphin-amide in guinea pig preparations (IC_{50} values of 14 × 10^{-6}, and 50 × 10^{-6} M, respectively) (Yoshikawa et al., 1986). Similarly, a recent report has shown that the μ-opioid peptide neocasomorphin-6 (YPVEPF) induced a significant rise in the transmembrane-associated mucin gene MUC4 but without effect on neither the expression of secreted MUC2 and MUC5AC nor the release of mucin-like glycoprotein (Plaisancié et al., 2015). The effect on mucin production by food protein hydrolysates and peptides appears to be mediated by more than one type of mechanism. It remains to be clarified in any of these α_{s1}-casein peptide fragments might stimulate mucin secretion and/or induce mucin gene overexpression other than MUC5AC.

In conclusion, various α_{s1}-casein fragments containing the tripeptide YFY, especially, ^{144}YFYPEL^{149}, ^{144}YFYPE^{148} have shown for the first time opioid activity in guinea pig ileum preparations. Other related fragments, ^{143}AYFYPEL^{149} and ^{144}YFY^{146} were able to exert this effect, but only when peptidase inhibitors were added to the preparation. This effect was antagonized by naloxone, which demonstrates the implication of opioid receptors. The susceptibility of these peptides to be hydrolysed by peptidases found in this tissue was demonstrated by HPLC-tandem mass spectrometry. Several of the resulting fragments lost the N-terminal Tyr residue, and therefore, the potential ability to interact with opioid receptors. Similarly to β-casomorphins, the assayed peptides exerted opioid activity on mouse vas deferens to a lower extent than in guinea pig ileum. The lack of activity of peptide ^{144}YFYP^{147} can be explained by the different position of the third intracellular loop at the receptor. In addition, it was found that interactions of Pro residue at ^{144}YFYPEL^{149} and ^{144}YFYP^{147} were established at different places of the receptor active site. The study of the MUC5AC expression in human goblet cells HT29-MTX has shown that bovine α_{s1}-casein ^{144}YFYPEL^{149} is the minimum fragment able to stimulate mucin production, whereas the smaller fragments ^{144}YFYPE^{148}, ^{144}YFYP^{147} and ^{144}YFY^{146} had no effect on MUC5AC expression, although it cannot be discarded activity on other mucin mRNA levels or on mucin secretion.

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FIGURE CAPTIONS

Figure 1. Inhibition of the electrically induced contractions in guinea pig ileum preparations at increasing concentrations (cumulative curve) of α_{s1}-casein fragment ^{143}AYFYPEL^{149} (A), α_{s1}-casein fragment ^{144}YFYPEL^{149} (B), α_{s1}-casein fragment ^{144}YFYPE^{148} (C), α_{s1}-casein fragment ^{144}YFYP^{147} (D), α_{s1}-casein fragment ^{144}YFY^{146} (E), β-casein fragment (β-casomorphin-7, ^{60}YPFPGPI^{66}) (F), and morphine (G). For clarity, food-derived peptides and morphine results are represented in the Y-axis up to 40 and 80% inhibition, respectively. Each point represents the mean % ± SEM (n = 6). Different letters denote statistically significant differences between concentrations ($P < 0.05$).

Figure 2. Inhibition of the electrically induced contractions in guinea pig ileum preparations in presence of the peptidase inhibitors (captopril, amastatin, and phosphoramidon) at increasing concentrations (cumulative curve) of α_{s1}-casein fragment ^{143}AYFYPEL^{149} (A), α_{s1}-casein fragment ^{144}YFYPEL^{149} (B), α_{s1}-casein fragment ^{144}YFYPE^{148} (C), α_{s1}-casein fragment ^{144}YFYP^{147} (D), and α_{s1}-casein fragment ^{144}YFY^{146} (E). Each point represents the mean % ± SEM (n = 6). Different letters denote statistically significant differences between concentrations ($P < 0.05$).

Figure 3. Mean residue fluctuations of the μ-opioid receptor and the α_{s1}-casein fragment ^{144}YFYPEL^{149} (A) and the α_{s1}-casein fragment ^{144}YFYP^{147} (B). Structures of opioid receptor and peptides are represented as ribbons where the most moving regions are thicker and coloured in red-orange, while static regions are thinner and colored in blue. Third loop between receptor residues 195-200 is marked with an arrow.

Figure 4. Final molecular dynamics structure of peptides ^{144}YFYPEL^{149} (A-D) and ^{144}YFYP^{147} (E-H) bound the μ-opioid receptor. Main residues involved in the interaction (showed as sticks) are highlighted in blue in the protein receptor, and those from the peptides in green. Interactions are shown by amino acid residue of each peptide ^{144}YFYPEL^{149} and ^{144}YFYP^{147}, respectively: N-

terminal Tyr (A and E); Phe (B and F); Tyr at third position (C and G); Pro-Glu-Leu from ^{144}YFYPEL^{149} (D) and C-terminal Pro from ^{144}YFYP^{147} (H).

Figure 1

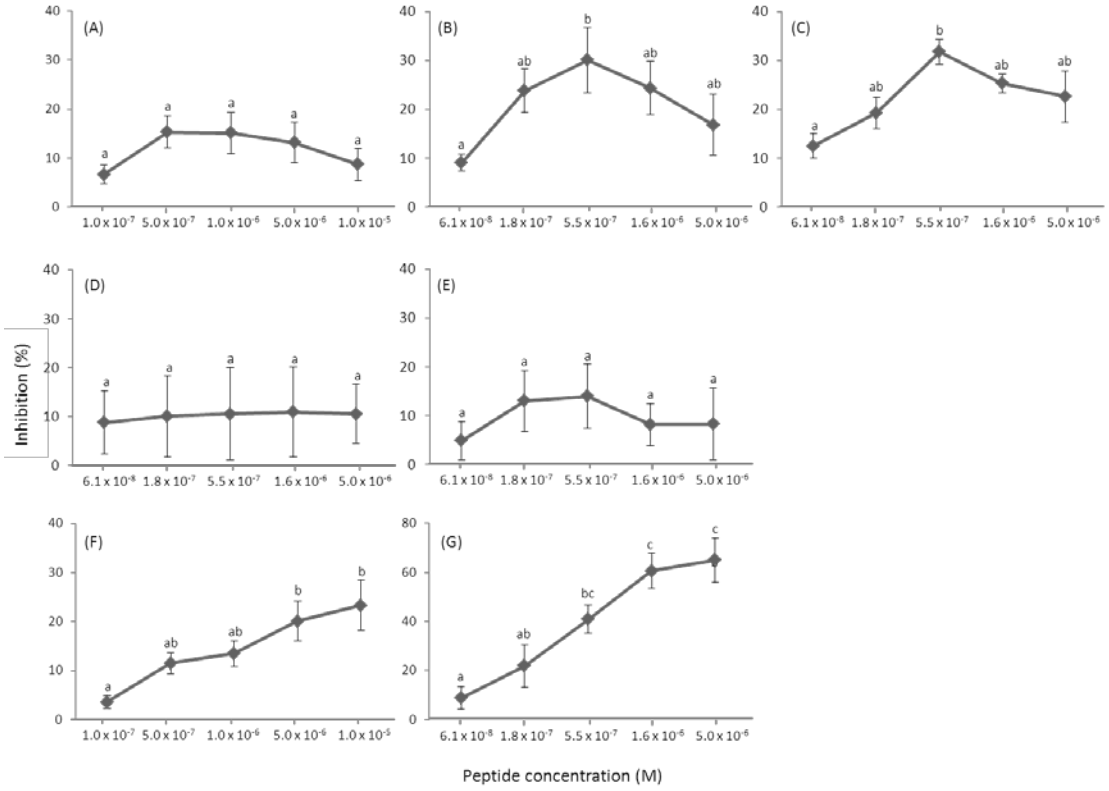

Figure 2

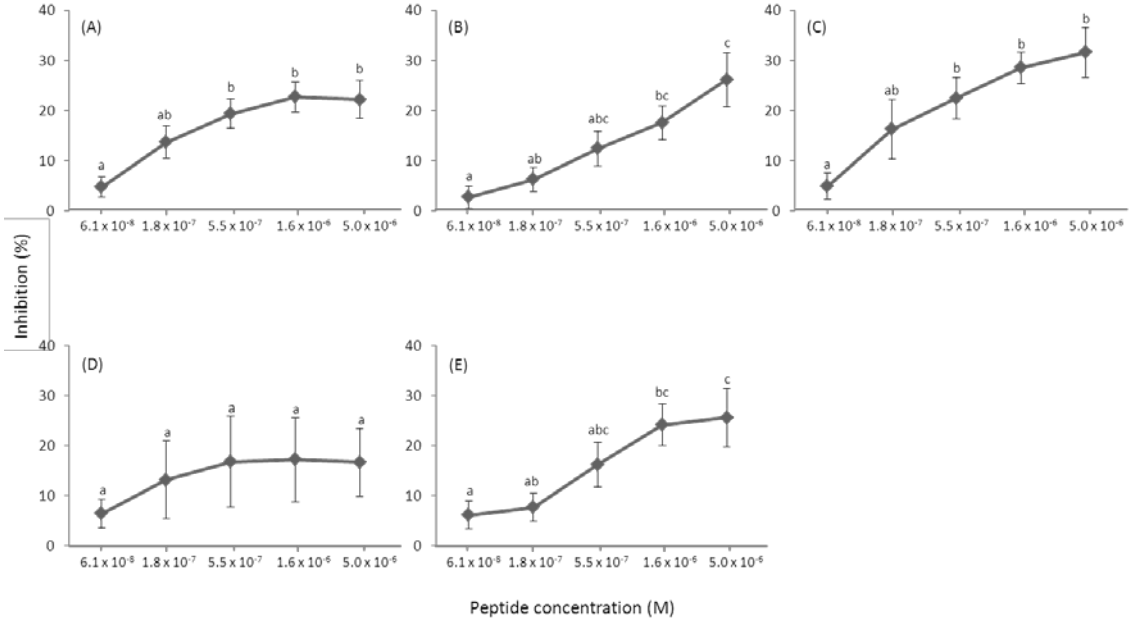
(A)
(B)
(C)
(D)
(E)
Inhibition (%)
Peptide concentration (M)
6.1 x 10⁻⁸
1.8 x 10⁻⁷
5.5 x 10⁻⁷
1.6 x 10⁻⁶
5.0 x 10⁻⁶

Figure 3

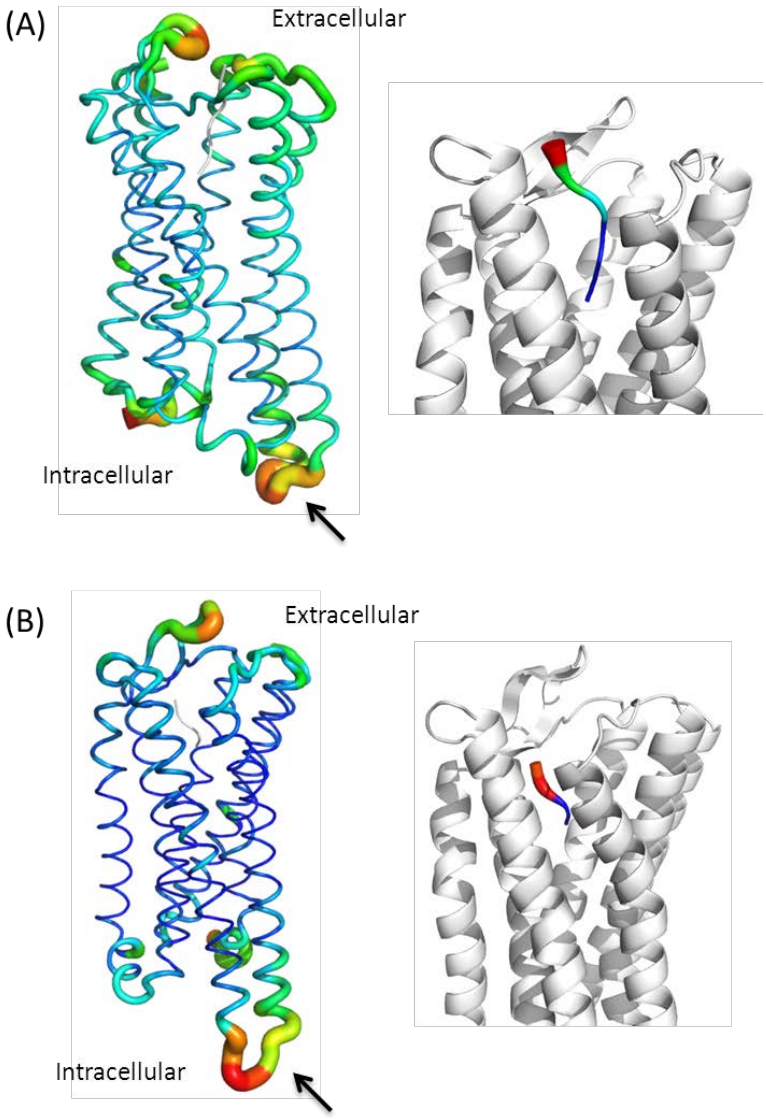
(A)
Extracellular
Intracellular
(B)
Extracellular
Intracellular

Figure 4